
Mutein of a bone morphogenetic protein and use thereof

The present invention is related to a mutein of a bone morphogenetic protein, nucleic acids coding therefor, methods for the production of such bone morphogenetic mutein, compositions, preferably pharmaceutical compositions comprising such mutein and the use of such mutein for the manufacture of a medicament.

The muteins according to the present invention are muteins of a bone morphogenetic protein. Bone morphogenetic proteins which are also referred to herein as BMP-2 like proteins, are one of two major groups forming the TGF- β superfamily. The other group of proteins belonging to said TGF- β superfamily apart from the BMP-2 like proteins are TGF- β -actin like proteins. The two groups of the TGF- β superfamily differ in their binding mechanisms. In general, the members of the TGF- β superfamily being structurally related polypeptide growth factors are involved in cellular processes, including cell proliferation, cell line determination, differentiation, mobility, adhesion and cell death. The factors are expressed according to a time and tissue specific pattern and are important for development, haemostasis and repair of nearly all tissues in eukaryotic organisms. These factors also account for an important part of intracellular signals which define the activity of a cell.

The signal transduction pathway of TGF- β has been elucidated in the more recent past. The signal transduction of TGF- β involves receptor serine kinases on the cell surface, and its substrates, namely the SMAD proteins, which are, upon phosphorylation migrating to the nucleus. The phosphorylated SMAD proteins activate transcription of the target gene in cooperation with DNA binding partners. The multifunctional activity of TGF- β and other members of the TGF- β superfamily seem to be based on the interaction of different receptors, SMAD proteins and DNA binding proteins. Any disturbance of this signal transduction pathway is the reason for a number of diseases in mammals and in man in particular. The members of the TGF- β superfamily share a number of structural features although the homology between said members is in some cases rather limited. For example, all of the proteins are dimers, typically are homodimers, i. e. comprised of two identical monomers. Also, the members of the TGF- β superfamily use cellular receptors which consist of two different types of serine kinase receptor chains, namely type I chain and type II chain.

The type I chain comprises a cytoplasmatic GS box and a serine kinase which activates SMAD-1 and SMAD-5 signal proteins if the type I chain is BRIA, BRIB or ARI. The type II chain activates a type I receptor serine kinase through phosphorylation of the GS box segment. The small receptor ecto domains of both the type I and type II chains, respectively, comprising about 120 to 150 amino acids only share a rather limited homology. Nevertheless, one common feature of all known receptor chains of the TGF- β superfamily are four conserved disulfide bridges; additional disulfide bridges and the position of some few amino acids seem to be characteristic for either type I or type II receptor proteins. The binding of BMP-2 like proteins to a type I chain occurs through the wrist epitope of the BMP-2 like proteins and to a type II chain through the "knuckle" epitope of the BMP-2 like proteins. The binding of these bone morphogenetic proteins to the respective receptor chains happens in a sequential manner at the cytoplasma membrane, whereby the particular sequence depends on the affinities of the bone morphogenetic protein to the individual receptor chains. For example, dissolved BMP-2 binds first to its high affinity type I chains, namely BMPR-IA, BMPR-IB and possibly also ActR-I, and subsequently assembles the low affinity type II receptor chain to form an active receptor complex. The assembling happens in the cytoplasma membrane.

Some amino acid substitutions in the wrist or in the knuckle epitope of BMP-2 result in inactive BMP-2 muteins which are no longer capable of activating the corresponding receptor. The inactive muteins which have the amino acid substitution in the knuckle epitope such as BMP-2 [A34D] are nevertheless able to bind to the type I chain similar to the wildtype BMP-2. However, the assembling of the type II chain in the membrane so as to form the active receptor complex comprising both the type I and type II chain is disturbed. Thus, this kind of muteins act as inhibitors of BMP-2 as they are competing with wildtype BMP-2 for the type I chain.

Apart from being involved in the signal transduction pathway involving a receptor comprising type I chains and type II chains, bone morphogenetic proteins and particularly BMP-2 like proteins interact with a group of modulator proteins. These modulator proteins are in turn involved in certain signal transduction pathways. Modulator proteins interacting with BMP-2-like proteins comprise, among others, members of the noggin protein family, the DAN protein family and the chordin protein family. These modulator proteins are known in the art and, for example, described in Avsian-Kretchmer, O. and Hsueh, A.J. (2004) Comparative genomic analysis of the eight-membered ring cystine knot-containing bone morphogenetic protein

antagonists. Mol Endocrinol, 18, 1-12, which describes BMP-modulator proteins, more particularly cystine-knot-containing BMP-modulator proteins. The noggin family comprising, e.g., noggin, is described by Canalis, E., Economides, A.N. and Gazzero, E. (2003) Bone morphogenetic proteins, their antagonists, and the skeleton. Endocr Rev, 24, 218-235; the chordin family comprising, among others, Chordin, Ventroptin, CTGF, CYR61, Crossveinless, Procollagen IIA, is, for example, described in Bork, P. (1993) The modular architecture of a new family of growth regulators related to connective tissue growth factor. FEBS Lett, 327, 125-130, Brigstock, D.R., Goldschmeding, R., Katsume, K.I., Lam, S.C., Lau, L.F., Lyons, K., Naus, C., Perbal, B., Riser, B., Takigawa, M. and Yeger, H. (2003) Proposal for a unified CCN nomenclature. Mol Pathol, 56, 127-128 and Garcia Abreu, J., Coffinier, C., Larrain, J., Oelgeschlager, M. and De Robertis, E.M. (2002) Chordin-like CR domains and the regulation of evolutionarily conserved extracellular signaling systems. Gene, 287, 39-47; and the DAN family comprising, for example Cer1, Cerberus, Dan, Dte, Gremlin, PRDC, Sclerostin is, e.g., described in Pearce, J.J., Penny, G. and Rossant, J. (1999) A mouse cerberus/Dan-related gene family. Dev Biol, 209, 98-110 and Balemans, W. and Van Hul, W. (2002) Extracellular regulation of BMP signaling in vertebrates: a cocktail of modulators. Dev Biol, 250, 231-250.

The problem underlying the present invention was thus to provide a mutein of bone morphogenetic proteins, particularly of BMP-2-like proteins which do not compete with the wildtype form of the respective bone morphogenetic proteins in terms of type I and type II serine kinase receptor chains mediated effects, but are still suitable to interact with modulator proteins such as those of the noggin protein family, the DAN protein family and the chordin protein family.

According to the present invention this problem is solved by the subject matter of the independent claims. Preferred embodiments may be taken from the dependent claims.

According to the present invention the problem is solved in a first aspect by a mutein of a bone morphogenetic protein, whereby the mutein comprises an amino acid substitution compared to the wildtype of the bone morphogenetic protein at the amino acid position corresponding to amino acid position 51 of human BMP-2.

In an embodiment the amino acid at the position corresponding to amino acid position 51 of human BMP-2 is leucine in the wildtype form of the bone morphogenetic protein and is preferably proline in the mutein.

In an embodiment the bone morphogenetic protein is selected from the group comprising hBMP-2, hBMP-4, hBMP-5, hBMP-6, hBMP-7, hBMP-8, hGDF-5, mGDF-6, mGDF-7, hBMP-10 and hGDF-2.

In an embodiment

- the bone morphogenetic protein is hBMP-2 and the position corresponding to amino acid position 51 of human BMP-2 is amino acid position 51;
- the bone morphogenetic protein is hBMP-4 and the position corresponding to amino acid position 51 of human BMP-2 is amino acid position 53;
- the bone morphogenetic protein is hBMP-5 and the position corresponding to amino acid position 51 of human BMP-2 is amino acid position 68;
- the bone morphogenetic protein is hBMP-6 and the position corresponding to amino acid position 51 of human BMP-2 is amino acid position 68;
- the bone morphogenetic protein is hBMP-7 and the position corresponding to amino acid position 51 of human BMP-2 is amino acid position 75;
- the bone morphogenetic protein is hBMP-8 and the position corresponding to amino acid position 51 of human BMP-2 is amino acid position 75;
- the bone morphogenetic protein is hGDF-5 and the position corresponding to amino acid position 51 of human BMP-2 is amino acid position 56;
- the bone morphogenetic protein is mGDF-6 and the position corresponding to amino acid position 51 of human BMP-2 is amino acid position 56;

- the bone morphogenetic protein is mGDF-7 and the position corresponding to amino acid position 51 of human BMP-2 is amino acid position 82;
- the bone morphogenetic protein is hBMP-10 and the position corresponding to amino acid position 51 of human BMP-2 is amino acid position 44; and
- the bone morphogenetic protein is hGDF-2 and the position corresponding to amino acid position 51 of human BMP-2 is amino acid position 45.

In an embodiment the wildtype of

- hBMP-2 comprises an amino acid sequence according to SEQ ID No. 1;
- hBMP-4 comprises an amino acid sequence according to SEQ ID No. 3;
- hBMP-5 comprises an amino acid sequence according to SEQ ID No. 5;
- hBMP-6 comprises an amino acid sequence according to SEQ ID No. 7;
- hBMP-7 comprises an amino acid sequence according to SEQ ID No. 9;
- hBMP-8 comprises an amino acid sequence according to SEQ ID No. 11;
- hGDF-5 comprises an amino acid sequence according to SEQ ID No. 13;
- mGDF-6 comprises an amino acid sequence according to SEQ ID No. 15;
- mGDF-7 comprises an amino acid sequence according to SEQ ID No. 17;
- hBMP-10 comprises an amino acid sequence according to SEQ ID No. 19; and
- hGDF-2 comprises an amino acid sequence according to SEQ ID No. 21.

In an preferred embodiment the wildtype of

- hBMP-2 is encoded by a nucleic acid according to SEQ ID No. 2;
- hBMP-4 is encoded by a nucleic acid according to SEQ ID No. 4;
- hBMP-5 is encoded by a nucleic acid according to SEQ ID No. 6;
- hBMP-6 is encoded by a nucleic acid according to SEQ ID No. 8;
- hBMP-7 is encoded by a nucleic acid according to SEQ ID No. 10;
- hBMP-8 is encoded by a nucleic acid according to SEQ ID No. 12;
- hGDF-5 is encoded by a nucleic acid according to SEQ ID No. 14;
- mGDF-6 is encoded by a nucleic acid according to SEQ ID No. 16;
- mGDF-7 is encoded by a nucleic acid according to SEQ ID No. 18;
- hBMP-10 is encoded by a nucleic acid according to SEQ ID No. 20; and
- hGDF-2 is encoded by a nucleic acid according to SEQ ID No. 22.

According to the present invention the problem is solved in a second aspect by a bone morphogenetic mutein, whereby the mutein is not binding to a first bone morphogenetic protein receptor and the mutein is binding to at least a modulator protein, whereby the modulator protein is selected from the group comprising the noggin protein family, the DAN protein family, the chordin protein family and the cysteine-knot-containing BMP modulator proteins.

According to the present invention the problem is solved in a third aspect by a bone morphogenetic mutein, which is preferably an embodiment of the first and the second aspect of

the present invention, comprising a pre-helix loop structure which interacts with a bone morphogenetic protein receptor, preferably a second bone morphogenetic protein receptor. It is to be understood that the term second bone morphogenetic protein receptor is used in order to distinguish this kind of receptor from another receptor which is referred to herein as first bone morphogenetic protein receptor. This terminology is introduced herein for reasons of reference only and does not make allusion to the binding of the protein and mutein respectively. Insofar the binding of the protein and mutein, respectively, preferably occurs to a single bone morphogenetic protein receptor only. Such single bone morphogenetic protein receptor can be either the first bone morphogenetic protein receptor or the second bone morphogenetic protein receptor.

In an embodiment according to the second and the third aspect the bone morphogenetic mutein according to claim 7 and 8, whereby the first and/or the second bone morphogenetic protein receptor is BRIA or BRIB.

In an embodiment according to the second and the third aspect the interaction is related to an amino acid residue, preferably amino acid residue Gln86 of BRIA or Gln 67 of BRIB.

According to the present invention the problem is solved in a fourth aspect by a bone morphogenetic mutein, which is in a preferred embodiment a bone morphogenetic protein according to the first, second and third aspect, comprising a pre-helix loop structure having an interaction with a second bone morphogenetic protein receptor, whereby the interaction of the pre-helix loop structure of the bone morphogenetic mutein with the second bone morphogenetic protein receptor is different from the interaction of the pre-helix loop structure of the wildtype bone morphogenetic protein with the second bone morphogenetic protein receptor.

In an embodiment according to any aspect the different interaction or the change is represented in refraction data, preferably such refraction data being acquired at room temperature to a resolution of at least about 2.7 Å.

In an embodiment according to any aspect the pre-helix loop structure is mutated compared to the wildtype of the bone morphogenetic protein.

In an preferred embodiment the amino acid corresponding to leucine at position 51 of the wildtype BMP-2 is mutated.

In another preferred embodiment the amino acid corresponding to leucine at position 51 of human BMP-2 is mutated to proline.

In an embodiment according to any aspect the bone morphogenetic mutein is a mutein of a bone morphogenetic protein selected from the group comprising hBMP-2, hBMP-4, hBMP-5, hBMP-6, hBMP-7, hBMP-8, hGDF-5, mGDF-6, mGDF-7, hBMP-10 and hGDF-2.

In an preferred embodiment the bone morphogenetic protein is BMP-2 or pro-BMP-2.

According to the present invention the problem is solved in a fifth aspect by a bone morphogenetic protein comprising an amino acid sequence according to any of SEQ ID Nos. 23 to 33.

In an embodiment the bone morphogenetic mutein is a bone morphogenetic mutein according to any aspect of the present invention.

According to the present invention the problem is solved in a sixth aspect by a nucleic acid coding for a bone morphogenetic protein and bone morphogenetic mutein, respectively, according to any aspect of the present invention and/or a complementary strand thereto.

According to the present invention the problem is solved in a seventh aspect by a nucleic acid comprising a nucleic acid sequence according to SEQ ID Nos. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 22, preferably coding for a bone morphogenetic mutein according to any aspect of the present invention, and/or a complementary strand thereto.

According to the present invention the problem is solved in an eighth aspect by a nucleic acid coding for a bone morphogenetic mutein according to any aspect of the present invention, whereby the nucleic acid would hybridize to the nucleic acid according to the sixth and seventh aspect but for the degeneracy of the genetic code, more preferably under stringent conditions.

According to the present invention the problem is solved in a ninth aspect by a vector comprising a nucleic acid according to the sixth, seventh and eighth aspect, whereby the vector is preferably an expression vector.

According to the present invention the problem is solved in a tenth aspect by a cell, preferably a mammalian cell, comprising a nucleic acid according to the sixth, seventh and eighth aspect and/or a vector according to the tenth aspect of the present invention.

According to the present invention the problem is solved in an eleventh aspect by a host organism, preferably a mammalian host organism and more preferably a non-human host organism comprising a cell according to the tenth aspect of the present invention.

According to the present invention the problem is solved in a twelfth aspect by a method for the production of a bone morphogenetic mitein according to any of the first to fifth aspect of the present invention, comprising the steps of

- a) cultivating a cell according to claim 25 in a cultivation broth and
- b) preparing the bone morphogenetic mitein from the cell and/or from the cultivation broth.

According to the present invention the problem is solved in a thirteenth aspect by a monoclonal antibody specifically binding to a bone morphogenetic mitein according to any of the first to fifth aspect of the present invention.

According to the present invention the problem is solved in a fourteenth aspect by a composition comprising a mitein according to any of the first to fifth aspect and/or a nucleic acid according to any of the sixth to eighth aspect of the present invention.

According to the present invention the problem is solved in a fifteenth aspect by a pharmaceutical composition comprising a mitein according to any of the first to fifth aspect or a nucleic acid according to any of the sixth to eighth aspect of the present invention, and a pharmaceutically acceptable carrier.

According to the present invention the problem is solved in a sixteenth aspect by the use of a bone morphogenetic mutein according to any of the first to fifth aspect and/or of a nucleic acid according to any of the sixth to eighth aspect of the present invention, for the manufacture of a medicament.

In an embodiment the medicament is for the treatment and/or prevention of a disease selected from the group comprising fibrotic diseases, wound healing, hypervascularization, vascular diseases, fractures, and osteoporosis.

In a preferred embodiment the fibrotic disease is selected from the group comprising renal fibrosis, hepatic cirrhosis, pulmonary fibrosis and chronic inflammation, preferably chronic inflammation associated with asthma.

In another embodiment the wound healing is related to keloid, cicatrization, and peritoneal obliteration.

In a further embodiment the hypervascularization is related to or associated with retinopathies, arteriosclerosis and/or tumors.

In a still further embodiment the fractures are non-healing fractures.

In another embodiment the disease is osteoporosis.

According to the present invention the problem is solved in a seventeenth aspect by the use of a morphogenetic protein, preferably a morphogenetic mutein according to any of the first to fifth aspect of the present invention as inhibitor to a BMP interacting protein.

In an embodiment the BMP interacting protein is selected from the group comprising the noggin protein family, the DAN protein family and the chordin protein family.

The present inventors have surprisingly found that the muteins according to the present invention are capable of inhibiting the activity of modulator proteins such as those of the noggin family, the DAN family and the chordin protein family while not interacting with the receptor of the corresponding bone morphogenetic protein which is the bone morphogenetic protein based on which the proteins are generated by substituting at least one amino acid, more particularly the BMP-2-like protein receptor. Due to these characteristics of the muteins according to the present invention, they may be applied to any organism where said modulator proteins are to be modulated, preferably inhibited thus abolishing or decreasing the inhibition exerted by said modulator proteins, while the other biological activity related to the bone morphogenetic protein which is mediated through said receptor chains is no longer present. This design of a mutein of a bone morphogenetic protein is clearly advantageous insofar as it can be used in the treatment or prevention of said modulator protein related or mediated diseases without triggering the undesired receptor mediated effects of this kind of bone morphogenetic protein. Insofar the mutein according to the present invention is "receptor-dead".

Also, the present inventors have surprisingly found that this particular characteristic of the bone morphogenetic muteins can be created by changing the pre-helix loop structure in the bone morphogenetic protein in its wildtype form. As used herein and if not indicated to the contrary, a bone morphogenetic protein is preferably a BMP-2-like protein. The pre-helix loop structure is preferably the structure of a bone morphogenetic protein which is, preferably in a secondary structure representation, the loop preceding the helix of the bone morphogenetic mutein. Preferably, the loop comprises four to ten, preferably six to eight, more preferably six amino acid residues. Taking the amino acid sequence of the human BMP-2 protein, the pre-helix loop structure extends from amino acid position 48 to amino acid position 53. As used herein, in preferred embodiments the pre-helix loop is any loop, and more preferably any sequence of amino acids which correspond to amino acids 48 to 53 of the human BMP-2, more preferably of the amino acid sequence according to SEQ. ID. NO. 1. Preferably, the pre-helix loop structure is mediating the interaction with a bone morphogenetic protein receptor, which is preferably referred to herein as type I bone morphogenetic protein receptor. Even more preferably, the type I bone morphogenetic protein receptor is BRIA. BRIA_{EC} is the extracellular domain of BMPR-IA and described in (see Kirsch, T., Sebald, W., Dreyer, M.K. (2000) Nat Struct Biol, 7, 492-496). BRIB_{EC} is the extracellular domain of BMPR-IB. ARI_{EC} is the extracellular domain of ActR-I.

The muteins according to the present invention are, due to the change in the structure, preferably secondary and tertiary structure as compared to the wildtype form of the bone morphogenetic protein no longer capable to interact with the type I bone morphogenetic protein receptor. The changes as preferably used herein, are those which are detectable by reference to the interaction of the wildtype form of the corresponding bone morphogenetic protein with said type I morphogenetic protein receptor. More particularly, it seems that in case of the type I bone morphogenetic protein receptor being the BRIA_{EC} the amino acid mostly influenced by the mutein according to the present invention is Gln86.

Also, the bone morphogenetic mutein according to the present invention comprises a pre-helix loop structure which is mediating the interaction with a type I bone morphogenetic protein receptor such as BRIA_{EC}, whereby the interaction is different from the interaction of the pre-helix loop structure of the wildtype form of the bone morphogenetic protein with the type I bone morphogenetic protein receptor. Any of these induced changes or different interaction pattern between the mutein and the respective second bone morphogenetic protein receptor are detected relative to the corresponding structure or interaction of the wildtype form of the bone morphogenetic protein. Such changes of either or both of said type I bone morphogenetic protein receptor and of the mutein can preferably be monitored by acquiring refraction data. In a more preferred embodiment, such refraction data are acquired at room temperature to a resolution of at least about 2.7 Å.

In a preferred embodiment, the bone morphogenetic protein is BMP-2. BMP-2 is known in the art and preferably comprises the amino acid sequence according to SEQ ID No. 1 as described herein and is preferably encoded by a nucleic acid comprising a nucleic acid sequence according to SEQ ID No. 2.

More particularly, the present inventors have also discovered that the particular characteristics of the muteins according to the present invention are conferred by an amino acid substitution, i. e. mutation, at the pre-helix loop structure, more particularly at the amino acid position of the bone morphogenetic proteins, preferably of the BMP-2 like proteins which corresponds to amino acid position 51 of human BMP-2, more preferably of the BMP-2 according to SEQ. ID. NO. 1. More particularly, the respective amino acid which is mutated in the mutein is the leucine in the

corresponding wildtype forms of the bone morphogenetic proteins. More preferably, the exchange is from a leucine residue to a proline residue. Due to the high homology and thus similar secondary and tertiary structure of the bone morphogenetic proteins, particularly of the BMP-2-like proteins, the particular position is similarly active and relevant, respectively, within the various members of this group of bone morphogenetic proteins so that the change, i. e. amino acid substitution is effective in any of said bone morphogenetic proteins and, thus, the respective muteins exhibit the characteristics outlined above. More preferably, the bone morphogenetic proteins are hBMP-2, hBMP-4, hBMP-5, hBMP-6, hBMP-7, hBMP-8, hGDF-5, mGDF-6, mGDF-7, hBMP-10 and hGDF-2 which are as such known in the art and all of which bind in a similar manner to BRIA_{EC}. However, the respective mutation turning them into corresponding muteins, i. e. having an amino acid substitution at their positions corresponding to amino acid position 51 of human BMP-2 distinguishes them from the prior art. The wildtype sequences of the aforementioned bone morphogenetic proteins are disclosed herein as SEQ ID No. 1 (hBMP-2), SEQ ID No. 3 (hBMP-4), SEQ ID No. 5 (hBMP-5), SEQ ID No. 7 (hBMP-6), SEQ ID No. 9 (hBMP-7), SEQ ID No. 11 (hBMP-8), SEQ ID No. 13 (hGDF-5), SEQ ID No. 15 (mGDF-6), SEQ ID No. 17 (mGDF-7), SEQ ID No. 19 (hBMP-10) and SEQ ID No. 21 (hGDF-2), whereby the corresponding amino acid sequences of the inventive muteins of said bone morphogenetic proteins differ in said single amino acid position compared to the wildtype and said amino acid sequences are those according to SEQ ID No. 23 (hBMP-2), SEQ ID No. 24 (hBMP-4), SEQ ID No. 25 (hBMP-5), SEQ ID No. 26 (hBMP-6), SEQ ID No. 27 (hBMP-7), SEQ ID No. 28 (hBMP-8), SEQ ID No. 29 (hGDF-5), SEQ ID No. 30 (mGDF-6), SEQ ID No. 31 (mGDF-7), SEQ ID No. 32 (hBMP-10) and SEQ ID No. 33 (hGDF-2). More particularly, said amino acid position 51 of human BMP-2 corresponds to position 53 in case of hBMP-4, to position 68 in case of hBMP-5, to position 68 in case of hBMP-6, to position 75 in case of hBMP-7, to position 75 in case of hBMP-8, to position 56 in case of hGDF-5, to position 56 in case of mGDF-6, to position 82 in case of mGDF-7, to position 44 in case of hBMP-10, and to position 45 in case of hGDF-2.

As used herein, a bone morphogenetic protein and thus the corresponding mutein which differs therefrom preferably only by the single amino acid substitution disclosed herein, can be present in the pre-pro-form, in the pro-form or as mature protein. This basic design is inherent to all of the bone morphogenetic proteins. In case of BMP-2, for example, the pre-pro-form comprises a total of 396 amino acids. The pre-sequence serves as a signal peptide and is responsible for the

transport of the nascent polypeptide chain into the endoplasmatic reticulum. After importation the protein folds into its native confirmation, whereupon disulfide bridges are formed. The pro-form of BMP-2 comprises all of the amino acids from position 20 to position 396. Accordingly, the pro-sequence comprises the amino acids from position 20 (Gly) to amino acid position 282 (Arg) of the human pre-pro-form of BMP-2. Finally, the mature BMP-2 comprises amino acids 283 to 396 of the pre-pro-form. The mature form is depicted herein also as SEQ ID No. 1.

It is within the present invention that the bone morphogenetic proteins and their corresponding muteins according to the present invention are present in the pre-pro-form, in the pro-form or in the mature form, whereby any signal peptide may act as pre-sequence, preferably of a bone morphogenetic protein, and any pro-sequence of any bone morphogenetic protein can be used as the pro-sequence of the particular bone morphogenetic protein, although it is more preferred that the pro-sequence attached to the N-terminus of the mature bone morphogenetic muteins correspond to the one attached to the N-terminus of the respective bone morphogenetic protein *in vivo*. The same also applies to the pre-sequence.

The term "bone morphogenetic protein" and "bone morphogenetic mutein", respectively, as preferably used herein, also comprises orthologues thereof. As used herein, an orthologue is a protein from another organism that fulfills the same genetic and physiological function as the reference protein. Also, in a more preferred embodiment, the term "bone morphogenetic protein" and "bone morphogenetic mutein" comprises any truncated protein and mutein, respectively. Preferably, such truncated protein and mutein lacks at least one amino acid residue, preferably at either the N terminus or the C terminus. Preferably the mutein lacks at least one domain. A bone morphogenetic mutein according to the present invention is thus also a truncated mutein as long as it still exhibits the characteristics outlined above, i. e. being "receptor-dead" and still suitable to interact with at least one modulator proteins, preferably a member of the noggin protein family, the DAN protein family and/or the chordin protein family.

It is also within the present invention that the mutein is further modified whereby such modification is preferably selected from the group comprising phosphorylation, pegylation and glycosylation.

It is to be understood that any of the nucleic acids according to the present invention can be present as DNA, RNA or any derivative thereof such as LNA or PNA. It is also within the present invention that the respective nucleic acid is present as a single strand or a double strand, either partially or completely. Finally, it is within the present invention that the term "nucleic acid sequence" also comprises the complement of such nucleic acid specified, for example as specified by the sequence according to a sequence identifiers, either alone or in combination with the other, essentially complementary strand.

The term "nucleic acid" as used herein also comprises any fragments of the nucleic acid as described herein, whereby preferably any such fragment comprises a length from about 19 to 30, more preferably 19 to 25 and most preferably 21 and 22 consecutive nucleotides. Even more preferably this kind of fragment of a nucleic acid is present as a double-stranded structure.

According to the present invention, the nucleic acid can also be a nucleic acid which hybridizes to any other form of nucleic acid disclosed herein, whereby such hybridization preferably occurs at stringent conditions. Such stringent conditions are, among others, described in Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning. A. Laboratory Manual, 2nd ed. pp. 9.47 – 9.55, Cold Spring Harbour Laboratory Press.

It will be acknowledged by the ones skilled in the art that starting from either the wildtype sequence of the bone morphogenetic protein or of the mutein thereof as described herein, it is possible to design a nucleic acid which is able to discriminate between the wildtype form of the bone morphogenetic protein and the mutein thereof. Preferably such discrimination is possible by increasing the stringency of the hybridization conditions.

It is also to be acknowledged by the ones skilled in the art that apart from the particular sequences disclosed herein, also different sequences, more preferably nucleic acid sequences, are comprised by the present invention as long as they are suitable to code for the muteins according to the present invention. Such sequence can be derived using the genetic code and factually any permutation of such sequence which arises due to the redundancy of the genetic code is within the present invention. About the particular amino acid substitution which is characteristic to the muteins according to the present invention where at the position of the bone morphogenetic proteins and muteins, respectively, corresponding to amino acid position 51 of human BMP-2

being proline rather than leucine it is to be acknowledged that the respective codon can be CCU, CCC, CCA or CCG.

The vector according to the present invention contains any of the nucleic acids as disclosed herein, including any of the fragments disclosed herein. Preferably, the vector is an expression vector. Such expression vector typically comprises a promotor or promotor-like structure and optionally further elements which are suitable to control the expression of the nucleic acid cloned into the vector such as to be controlled by such promotor and other controlling elements. Preferably, the expression vector is a prokaryotic expression vector or an eukaryotic expression vector or a respective shuttle vector. The particular design of expression vectors of this type is known to the ones skilled in the art.

The cell according to the present invention is either a prokaryotic cell or an eukaryotic cell. As prokaryotic cell, in principle, *E. coli*, *B. subtilis* or *S. carnosus*, is used for the expression of the nucleic acid coding for a bone morphogenetic mutein according to the present invention, or a fragment or derivative thereof.

An eukaryotic cell as used herein is preferably a yeast cell or a mammalian cell. More preferably, the mammalian cell is a cell from mouse, rat, guinea-pig, pig, monkey or human being.

The host organism as used herein is preferably any of the cells according to the present invention or a multi-cellular organism. Such multi-cellular organism is preferably a mammal, more preferably a non-human mammalian. A preferred non-human mammal is selected from the group comprising mouse, rat, guinea-pig, sheep, rabbit and pig.

In the method according to the present invention for the production of a bone morphogenetic mutein according to the present invention, preferably a cell according to the invention is cultivated. Such cultivation is preferably performed in a cultivation broth. A cultivation broth is preferably a fermentation broth or any other solution which is suitable for the cultivation and more preferably for the propagation of the cell to be cultivated. Cultivation broths are known to the ones skilled in the art. Preferably upon cultivation and/or propagation of the cell the mutes according to the present invention may be prepared from the cultivation broth. Preparation from

the cultivation broth, as used herein, comprises the preparation from the cell which may contain the mutein according to the present invention either intracellularly or otherwise attached to the cell, or may comprise the isolation or preparation of the mutein according to the present invention from the cultivation broth. Methods for preparing or isolating the mutein according to the present invention are known to the ones skilled in the art.

In a further aspect the present invention is related to a monoclonal antibody which is directed against a mutein according to the present invention. Preferably, the antibody is a monoclonal antibody. It is to be acknowledged by the one skilled in the art that it is possible to prepare an antibody, preferably a monoclonal antibody which is specifically binding to a bone morphogenetic mutein according to the present invention.

Preferably, the term "specifically binding" means that the antibody is not binding to the wildtype form of the bone morphogenetic protein to the same extent or following the same binding characteristics as to the mutein, whereby at least the binding constant of the mutein compared to the corresponding wildtype form of the bone morphogenetic protein differs by a factor of at least 2, preferably by a factor of at least 5 and most preferably by a factor of at least 10.

In a further aspect the present invention is related to a composition comprising either a mutein according to the present invention or a nucleic acid according to the present invention. Such composition may comprise apart from the protein and/or the nucleic acid a carrier. Preferably such carrier is selected from the group comprising solid phases, aqueous phases and lipid phases. Lipid phases as used herein are preferably lipids or a lipid compositions complexing or encompassing the nucleic acid and the mutein, respectively. Preferred lipid compositions insofar are liposomes as known to the ones skilled in the art. Aqueous phases are preferably selected from the group comprising water and aqueous buffer solutions and hyaluronic acid. Solid phases are preferably tricalcium phosphat, polylactides, collagen and insoluble collagen bone matrix.

In a further aspect the present invention is related to a pharmaceutical composition. Such pharmaceutical composition comprises a mutein according to the present invention or a nucleic acid according to the present invention, or both, in addition to a pharmaceutically acceptable carrier. A pharmaceutically acceptable carrier is preferably either a liquid or a solid carrier. Suitable liquid carriers are, among others, water, aqueous solutions, more preferably buffers, or

lipids or lipid compositions. Preferred solid carriers comprise, among others, sugar, cellulose and starch. It is to be acknowledged that those skilled in the art, preferably in the art of formulations, are aware of further pharmaceutically acceptable carriers and carrier material, respectively. In a preferred embodiment the pharmaceutical composition comprises a further pharmaceutically active agent.

In a further aspect the present invention is related to the use of a bone morphogenetic mutein according to the present invention and/or of a nucleic acid according to the present invention for the manufacture of a medicament.

It will be acknowledged by the ones skilled in the art that based on the surprising findings of the present inventors, namely that the muteins of bone morphogenetic proteins and more particularly of BMP-2 like proteins according to the present invention do not exhibit the receptor mediated effects of such bone morphogenetic proteins, i. e. and are thus "receptor-dead", while still interacting with the modulator proteins with which the wildtype form of the respective bone morphogenetic protein interact. Insofar, the muteins according to the present invention and nucleic acids coding therefor, can be applied to a patient in need thereof without running into the risk that the known undesired effects of bone morphogenetic proteins will occur, thus outweighing the benefits of interacting with the modulator proteins inhibiting their inhibitory activity. For example, undesired activities of BMP-2 are, among others, promoting tumor growth. Because of this, the medical use of bone morphogenetic proteins, including any muteins thereof of the prior art, has been limited to local administration such as in bone regeneration. However, the muteins according to the present invention being devoid of those effects of bone morphogenetic protein activities particularly mediated through the interaction with their receptors allow for the very first time the medical application of this class of pharmaceutically active compounds.

A further aspect of the present invention is related to a method for the treatment of a patient suffering from or being in a condition to develop any of the diseases described herein for which the medicament according to the present invention may be used. Such method comprises the administration of a mutein or a nucleic acid coding therefor, to the patient. Preferably, the patient is a mammal, more preferably the patient is a human being.

Due to the mode of action of the muteins according to the present invention, they can be readily used for various diseases, namely those diseases which can be treated or prevented by providing for an undisturbed interaction between the bone morphogenetic proteins and corresponding muteins according to the present invention, and any modulator proteins. Preferably, the modulator proteins are known in the art and are selected from the group comprising cystine knot-containing bone morphogenetic proteins, more particularly the cystine knot-containing BMP modulator proteins, the noggin family, the chordin family, and the DAN family. The cystine knot-containing BMP modulator proteins are, for example, described in Avsian-Kretchmer, O. and Hsueh, A.J. (2004) Comparative genomic analysis of the eight-membered ring cystine knot-containing bone morphogenetic protein antagonists. *Mol Endocrinol*, 18, 1-12. The noggin family comprising, e. g., noggin, is described by Canalis, E., Economides, A.N. and Gazzero, E. (2003) Bone morphogenetic proteins, their antagonists, and the skeleton. *Endocr Rev*, 24, 218-235; the chordin family comprising, among others, Chordin, Ventroptin, CTGF, CYR61, Crossveinless, Procollagen IIA, is, for example, described in Bork, P. (1993) The modular architecture of a new family of growth regulators related to connective tissue growth factor. *FEBS Lett*, 327, 125-130, Brigstock, D.R., Goldschmeding, R., Katsube, K.I., Lam, S.C., Lau, L.F., Lyons, K., Naus, C., Perbal, B., Riser, B., Takigawa, M. and Yeger, H. (2003) Proposal for a unified CCN nomenclature. *Mol Pathol*, 56, 127-128 and Garcia Abreu, J., Coffinier, C., Larrain, J., Oelgeschlager, M. and De Robertis, E.M. (2002) Chordin-like CR domains and the regulation of evolutionarily conserved extracellular signaling systems. *Gene*, 287, 39-47; and the DAN family comprising, for example, Cer1, Cerberus, Dan, Dte, Gremlin, PRDC, Sclerostin is, e. g., described in Pearce, J.J., Penny, G. and Rossant, J. (1999) A mouse cerberus/Dan-related gene family. *Dev Biol*, 209, 98-110 and Balemans, W. and Van Hul, W. (2002) Extracellular regulation of BMP signaling in vertebrates: a cocktail of modulators. *Dev Biol*, 250, 231-250. It is to be acknowledged that these modulator proteins are known to have an inhibitory or stimulatory effect on pathways and events in a cell which, when biased, particularly when not inhibited themselves, tend to have an inhibitory effect on *in vivo* processes resulting in some diseases or a diseased condition. Therefore, without wishing to be bound by any theory, the muteins according to the present invention which may still interact with said modulator proteins and can thus inhibit their modulatory effect allowing that the process otherwise inhibited or stimulated by the modulator proteins can be performed at all or at a higher or lower level compared to the extent observed in the presence of the modulator proteins alone.

Particularly preferred diseases which can be treated by the muteins and the nucleic acids coding therefor, according to the present invention are fibrotic diseases. Particularly preferred diseases which may be treated according to the present invention are renal fibrosis, hepatic cirrhosis, pulmonary fibrosis and chronic inflammation, more preferably chronic inflammation associated with asthma and, therefore, also the treatment of asthma is comprised by the present invention.

Again without wishing to be bound by any theory, CTGF belonging to the Chordin-family interacts with BMP-2 and thus with the muteins according to the present invention. CTGF is mediating a pro-fibrotic activity of TGF- β . More particularly, TGF- β is stimulating the production of proteins of the extracellular matrix and inhibits the degradation of this kind of proteins. Normally, this process is an integral part of the healing of tissue. In case of chronic diseases, however, such as inflammatory diseases and more particularly chronic inflammation, TGF- β activity is exuberant thus leading to tissue fibrosis, particularly excessive tissue fibrosis (Branton, M.H. and Kopp, J.B. (1999) TGF-beta and fibrosis. *Microbes Infect*, 1, 1349-1365). These various fibrotic diseases can thus be inhibited by the administration of a mutein according to the present invention.

A further kind of diseases which may be treated according to the present invention is wound healing. More preferably, the form of this medical condition, i. e. wound healing, are keloid, cicatrization, and peritoneal obliteration. A particular group of patients which can be treated according to the present invention are patients who undergo or have undergone a glaucoma surgery which preferably goes along with intense cicatrization affecting eye vision. Again, without wishing to be bound by any rational, the exuberant cicatrization seems to be mediated through CTGF and CYR61 (Branton, M.H. and Kopp, J.B. (1999) TGF-beta and fibrosis. *Microbes Infect*, 1, 1349-1365) so that an inhibition of these modulator proteins through the muteins according to the present invention is a suitable means to avoid cicatrization and thus to treat the respective condition and disease, respectively.

Another disease or condition to be treated according to the present invention is hypervasculization. More particularly, the term "hypervasculization" comprises any disease which is caused, related to or associated with hypervasculization. Preferably this kind of diseases are tumors, retinopathies and arteriosclerosis. Insofar the present invention is also related to the use of the muteins according to the present invention for the treatment of tumors,

retinopathies. Preferably, the tumors are solid tumors. Also, vascular diseases, such as preferably arteriosclerosis, can be treated according to the present invention. Again, without wishing to be bound by any theory it seems that solid tumors require a sufficient vascularization in case they grow beyond a volume of 1 to 3 mm³. A sufficient blood supply is a limiting step during growth and progression of a tumor. According to the current understanding of the present inventors, CYR61 is involved in tumor angiogenesis. More particularly, CYR61 and CTGF, respectively, are understood as being involved in angiogenic and fibrogenous progresses which are involved in progressive vascular occlusion (Brigstock, D.R. (2002) Regulation of angiogenesis and endothelial cell function by connective tissue growth factor (CTGF) and cysteine-rich 61 (CYR61), *Angiogenesis*, 5, 153-165). Thus by interacting with said target molecules, i. e. CYR61 and CTGF, the muteins according to the present invention are suitable for the prevention and treatment of the respective diseases.

A further disease and condition, respectively, which can be treated according to the present invention are fractures, preferably non-healing fractures. Again without wishing to be bound by any theory it seems that noggin and CTGF are BMP-modulating proteins involved in the healing of fractures. More particularly, noggin and CTGF inhibit and terminate bone formation (Yoshimura, Y., et al., (2001), Colocalization of noggin and bone morphogenetic protein-4 during fracture healing, *J Bone Miner Res*, 16, 876-884). Thus inhibiting the effect of noggin and CTGF by, for example, competitive inhibition with the interaction partners of said targets, the muteins according to the present invention affect a treatment of this condition.

Osteoporosis is another disease which can be treated according to the present invention. Again without wishing to be bound by any theory, it seems that in connection with this disease the target is sclerostin. Sclerostin inhibits the bone forming processes in an organism. People suffering from an inactivation of the sclerostin gene (SOST) show a continuous increase in bone density which may reach up to three times of the standard value. Insofar any compound inhibiting sclerostin would be a suitable means for the treatment of conditions going along with reduced bone density such as, for example, osteoporosis (Balemans, W. and Van Hul, W. (2002), Extracellular regulation of BMP signaling in vertebrates: a cocktail of modulators, *Dev Biol*, 250, 231-250). Insofar given the fact that the muteins according to the present invention interact with sclerostin and thus inhibit this compound, they are suitable for the treatment of this kind of diseases and conditions, respectively, more preferably of osteoporosis.

The present invention is further illustrated by the figures and examples from which further features, embodiments and advantages may be taken, wherein

Fig. 1 (A) shows a ribbon sketch of the complex structure of BMP-2 and BRIA_{EC};

Fig. 1 (B) shows a surface representation in an “open book” view with the receptor BRIA being rotated 180° around the y-axis, of the interface of BMP-2 and BRIA_{EC};

Fig. 1 (C) shows a surface representation of the interface of BMP-2 and BRIA_{EC};

Fig. 2 shows a zoom-in into the interface of the BMP-2-BRIA interaction;

Fig. 3 (A) shows a stereo view of the pre-helix loop segment showing the crystal structure of the BMP-2 L51P;

Fig. 4 shows the biological activity of BMP-2 proline muteins expressed as induction of alkaline phosphatase (ALP) activity measured in ATDC5 (A) and C2C12 (B) cells in response to BMP-2 and BMP-2 muteins;

Fig. 5 shows the release of noggin inhibition by BMP-2 proline mutein L51P;

Fig. 6 shows an alignment of BMP-2 like proteins which upon exchanging the amino acid residue at the position corresponding to amino acid position 51 of human BMP-2 from leucine to preferably proline form preferred embodiments of the muteins according to the present invention;

Fig. 7 shows sensograms of the interaction between BMP proline muteins and (A) BRIA_{EC}, (B) BRIB_{EC}, or (C) Activin receptor II ectodomain and (D) interaction between BMP-2 and BRIA_{EC} muteins, whereby all ligands were present at 120 nM concentration and the sensorgrams were normalised by setting resonance units (RU) calculated for saturating analyte BMP-2 concentration as 100%; and

Fig. 8 shows structural differences between BMP-2 wild type and BMP-2 L51P, whereby the C_α -atoms of both structures were superimposed and the in-between distances are shown and the region for the pre-helix loop is highlighted in grey.

Example 1: Materials and Methods

Preparation of proteins

Preparation of BMP-2 mutein and BMP receptor ectodomain proteins was performed as described previously (Kirsch et al., 2000b; Ruppert et al., 1996). Variants for BMP-2 or BRIA_{EC} were obtained by recombinant PCR (Kirsch et al., 2000a). BMP-2 wildtype and mutein protein used for crystallization was purified by cation exchange chromatography using an EMD SO₃⁻ column (Merck). Fractions were analyzed by SDS-PAGE and pooled fractions were dialyzed against water and freeze-dried. Homogeneity of the proteins was checked by ESI FT-CR mass spectroscopy and analytical reversed-phase HPLC. Binding activity of the refolded BMP-2 muteins was assessed by measurement of the binding affinity for BMP type I and II receptor using BIACore methodology. Preparation of the BMP-2 – BRIA_{EC} complex for structure analysis was performed as published (Kirsch et al., 2000b).

Crystallisation of the proteins

For crystallisation, BMP-2 D53P and BMP-2 L51P were dissolved in water at a concentration of 5 and 8 mg ml⁻¹, respectively. BMP-2 L51P was crystallised by the hanging drop vapour diffusion method at room temperature from 15% t-butanol, 0.1 M lithium sulphate and 0.1 M sodium citrate pH 5.0. Crystallisation of the complex of BMP-2 - BRIA_{EC} was performed basically as published (Kirsch et al., 2000b) however crystals for measurement at 100 K were obtained by co-crystallising using glucose as cryo-protectant. High quality crystals for data acquisition grew from 0.75 M sodium acetate, 0.1 M imidazole pH 7.0 and 30% glucose at 10°C using a protein concentration of 11 mg ml⁻¹.

X-ray data acquisition

Data for the complex of BMP-2 – BRIA_{EC} were obtained from a single crystal at 100 K at the beamline X06SA PX at the Swiss Light Source (Paul Scherrer Institute, Switzerland). Two datasets with 90° rotation of the crystal (1° per frame) were measured, a high-resolution set with 10 s exposure for 1° yielded a maximum resolution of 1.9 Å, low-resolution data was obtained by recording with 1s exposure time per degree. The data were processed and integrated using the software MOSFLM version 6.2.1 (Leslie, 1992), the two datasets were merged (overlap: 20 - 5.0 Å for low-resolution, 20 - 1.9 Å for high-resolution) and scaled using the software SCALA CCP4 version 4.2.1 (Collaborative Computational Project, 1994; Evans, 1993), a summary for the processing statistics is given in table A supplement. Diffraction data for the BMP-2 mutein L51P were acquired at room temperature on a home-source consisting of a Rigaku RU-300 (50 kV, 100 mA, 0.3 mm filament), Osmic ConfocalBlue optics and a Rigaku R-AXIS IV++ imageplate system. The exposure time was set to 5 min per 0.5° rotation, crystals diffracted up to 2.5 Å. An acquisition strategy was determined using the software CrystalClear (Rigaku) to minimize radiation damage, however to realize reasonable completeness, data from two crystals was collected, merged and scaled using the software MOSFLM and SCALA (Evans, 1993; Leslie, 1992).

Structure determination

The structure of the complex of BMP-2 – BRIA_{EC} was refined by using the lower resolution structure (PDB entry 1ES7) as a start model. The BMP-2 – BRIA_{EC} complex crystallises in the space group P6₅, with the asymmetric unit holding a BMP-2 dimer and two BRIA_{EC} molecules. Due to slight changes in the cell constants, a molecular replacement step consisting of a rotation and translation search followed by a rigid body refinement was performed using the software CNS 1.1 (Brünger et al., 1998). The program REFMAC5 (Murshudov et al., 1997) was subsequently used for refinement followed by manual rebuilding of the coordinates using the software QUANTA2000 (Accelrys). The resolution was gradually increased to a final resolution range of 20 to 1.9 Å. One TLS group was defined for each chain, i.e. BMP-2 monomers and each BRIA_{EC} molecule to account for anisotropy in the data (Winn et al., 2001). The progress of refinement was monitored by cross-validation using a test data set comprising of 5 % of the

reflections (Brünger, 1992). Since the complex structure is principally symmetrical, we tried to employ non-crystallographic symmetry restraints, however in the subsequent refinement steps the R_{free} could not be lowered without the NCS restraints being violated. Therefore, no NCS restraints were used throughout the refinement. In the final round of refinement $f_{\text{obs}}-f_{\text{calc}}$ difference electron density maps were used to identify 185 water molecules. The final minimization cycle yielded an R-factor of 20.7 and 22.9 for R_{free} . The structure analysis of BMP-2 L51P followed the protocol for the BMP-2 - BRIA_{EC} complex however CNS 1.1 was used for simulated annealing and energy minimization. The structure of BMP-2 wild type (PDB entry 3BMP) served as a start model. Manual rebuilding of the model was based on σ_A -weighted $2f_{\text{obs}}-f_{\text{calc}}$ and $f_{\text{obs}}-f_{\text{calc}}$ electron density maps, in the final round of refinement 13 water and two MPD molecules were added. The R-factor for the final structure of BMP-2 L51P is 21.5 and 23.5 for R_{free} .

Interaction analysis

Proteins were biotinylated and immobilised to streptavidin-coated sensor chip CM5 (Kirsch et al., 2000a). The extracellular domain of BRIA, BRIB and ARII were prepared as described (Kirsch et al., 2000a). Noggin/Fc, Chordin and Gremlin were obtained from R&D systems (Wiesbaden-Nordenstadt). Interaction with analyte was measured on a BIACore 2000 system (Kirsch et al., 2000a). Evaluation of sensorgrams (Biaevaluation software 2.0) yielded kinetic constants for complex formation (k_{on}) and dissociation (k_{off}). 6 to 12 measurements yielded mean values with a mean standard deviation of 35% for k_{off} and 12% for k_{on} . Apparent dissociation constants K_D were either calculated as $K_D = k_{\text{off}}/k_{\text{on}}$ or by evaluating dose dependency of equilibrium binding. Special conditions had to be applied to regenerate free Noggin after BMP-2 binding. The remaining complex could be only dissociated at acidic pH 3 in the presence of 1M sodium chloride and 6M Urea. Fortunately, the protein retained native binding properties after this harsh treatment as established by independent single cycle experiments employing immobilisation of the Noggin-Fc fusion protein to sensor-fixed protein A.

Biological activity in cell lines

Alkaline phosphatase (ALP) activity was determined in serum-starved ATDC5 (Riken Bioresource Center, Cell number RCB0565) and C2C12 (ATCC Number CRL-1772) cells (Kirsch et al., 2000a).

Example 2: Hydrogen bonding pattern in the BMP-2-BRIA_{EC} complex

The high resolution structure of the BMP-2 - BRIA_{EC} complex contains one complete BMP-2 dimer bound to two BRIA ectodomains in the asymmetric unit (Fig. 1A). Applying strict non-crystallographic symmetry failed showing that the symmetry of the ligand-receptor complex is not perfect. However, differences between the two segments (BMP-2 dimer and one BRIA_{EC} molecule) are small with an r.m.s. deviation of 0.9 Å for all atoms and 0.4 Å for all C_α-atoms. A detailed analysis shows that the structure of the two BMP-2/BRIA_{EC} interfaces is the same. All dissimilarities between the non-crystallographic symmetry related molecules are due to differences in the packing environment in the crystal lattice. The high temperature factor in these segments suggests that all the differences might be due to backbone flexibility. Thus, structural variations in the symmetry-related protein segments do not reflect real dissimilarities between the individual molecules but rather possible conformations and dynamics of the BMP-2 and BRIA polypeptide chains.

The BMP-2/BRIA interface represents a new paradigm for a growth factor/receptor interface. The binding epitope of BMP-2 is highly hydrophobic with almost 60% of the total buried surface area of 2310 Å² built by hydrophobic residues (Fig. 1b). Compared with binding epitopes of other growth factors, like human growth hormone (de Vos et al., 1992), interleukin-4 (Hage et al., 1999), erythropoietin (Syed et al., 1998) or interleukin-6 (Boulanger et al., 2003), this level of hydrophobicity is quite high. An extremely hydrophobic contact side seems to be characteristic for members of the TGF-β superfamily, as shown in the complex structures of BMP-7 bound to Activin receptor II ectodomain (Greenwald et al., 2003), TGF-β3 in complex with TGF-β receptor II (Hart et al., 2002), and Activin A bound to Activin receptor IIB (Thompson et al., 2003). In contrast, the binding epitope of BRIA_{EC} is much less hydrophobic

(30%) due to a stretch of polar and charged residues running across the binding interface (Fig. 1B).

The higher resolution of 1.9 Å of the refined structure of the BMP-2 - BRIA_{EC} complex allows for a more detailed analysis as was possible for the previous structure at lower resolution. In each BMP-2/receptor interface 10 intermolecular hydrogen bonds (H-bonds) are identified by the program HBPLUS (McDonald and Thornton, 1994) (Table 1). BMP-2 provides 5 and the receptor 4 main chain atoms to hydrogen bonding. The atoms engaged in H-bonds cluster into two structural elements as depicted in table 1: on the ligand BMP-2 the pre-helix loop consisting of residues Phe 49 to Asn 59, and on the receptor BRIA the short α -helix and flanking sequences (Asp 84 to Arg 97). Two hydrogen bonds, BMP-2 Asp 53 (N) – BRIA_{EC} Cys 77 (O) and BMP-2 Ser 69 (O) – BRIA_{EC} Gln 94 (N), are formed between main chain atoms only, two hydrogen bonds occur between side chains only and 4 mixed hydrogen bonds comprise side chain and main chain atoms (Table 1). Eight of these ten hydrogen bonds have donor - acceptor distances (2.8 ± 0.14 Å) and ϕ bond angles (137 ± 11 °) as expected for bona fide hydrogen bonds (Xu et al., 1997). However, a classification of the strength of an individual hydrogen bond based purely on a geometrical evaluation of the bond parameters seems not possible for this complex.

Table 1: Geometry of H-bonds in the BMP-2 - BRIA interface.

BRIA _{EC}	BMP-2	Distance Å	Angle NOC ^{b)}	H-bond ^{c)}	Mutein ^{d)}
T55 (OG1)	D53 (OD2)	2,73 (2,79)	127 (127)	SC-SC	<i>D53A/D53P</i>
C77 (O)	D53 (N)	2,88 (2,86)	136 (131)	MC-MC	<i>D53P</i>
G81 (N)	N59 (OD1)	3,27 (3,30)	151 (152)	MC-SC	<i>N59A</i>
D84 (OD2)	Y103 (OH)	2,79 (2,64)	147 (155)	SC-SC	<i>Y103A</i>
Q86 (OE1)	L51 (N)	2,75 (2,76)	127 (127)	SC-MC	<i>Q86A/L51P</i>
Q86 (NE2)	L51 (O)	2,97 (3,02)	134 (133)	SC-MC	<i>Q86A</i>
D89 (O)	W28 (NE1)	2,88 (2,87)	135 (123)	MC-SC	<i>W28F</i>
S90 (OG)	V26 (O)	2,53 (2,62)	128 (134)	SC-MC	<i>S90A</i>
Q94 (N)	S69 (O)	2,87 (2,88)	151 (153)	MC-MC	<i>Q94P</i>
R97 (NZ2)	S69 (OG)	3,10 (3,05)	90 (96)	SC-SC	<i>S69R</i>
mean value ^{a)}		2,8 (2,78)	137 (137)		
S.D.		0,14 (0,14)	10 (13)		

The numbers in bracket represent the distances of donor-acceptor atom and angles in the second interface related by non-crystallographic symmetry.

- a) H-bonds in the shaded rows were excluded from above geometrical statistics;
- b) N-O-C are the Donor-Acceptor atoms; from statistics (Xu et al., 1997) this angle is $149^\circ \pm 15^\circ$ for MC-MC hydrogen bonds and $129^\circ \pm 18^\circ$ for SC-MC and SC-SC H-bonds;
- c) MC (main chain) and SC (side chain) donor/acceptor atoms;
- d) Analysed H-bond disrupting substitutions in BMP-2 (italic) and BRIA (bold).

In order to explore whether functionally important H-bonds can be predicted from the interface structure, the residual fractional accessibility was calculated (Fig. 1C). This residual accessibility in the complex correlates with the location of a residue towards the core or towards the periphery of the interface (Chakrabarti and Janin, 2002). Eight of the ten interfacial H-bonds occur between residues located at the periphery. Only the two H-bonds connecting receptor Gln 86 to the BMP-2 main chain of Leu 51 amide and carbonyl is completely buried upon complex formation (Fig. 1C). Consequently, this interaction might represent a hot spot of binding (Bogan and Thorn, 1998), whereas the other H-bonds and hydrophobic interactions might contribute not or only marginal to binding affinity.

Examination of water molecules in the interface reveals that the desolvation of the binding epitope is rather efficient compared to other protein-protein interfaces. Only four water molecules are completely buried in each ligand-receptor interface upon complex formation. The low temperature factors of these buried water molecules (i.e. values for these buried water molecules range from 40 to 50 \AA^2 with protein atoms in close proximity having B-factors of 32 \AA^2) suggest that exchange with external water might be slow. Surprisingly, these water molecules are in close proximity of the hot spot BRIA_{EC} Gln 86. Three water molecules are located on the “membrane-distal side” of BRIA_{EC} Gln 86 near to the side chain amide nitrogen atom. These hydrogen-bonded water molecules fill a larger cleft in the interface with BRIA_{EC} Lys 97 blocking the entrance to this cavity. A single isolated water molecule is located in a hollow space next to the side chain carbonyl oxygen atom of BRIA_{EC} Gln 86. This cavity is blocked from the outside by the residues Phe 49 and Pro 50 of BMP-2. The incomplete

desolvation of receptor Gln 86 during BMP-2 binding possibly supports the formation of a strong hydrogen bond in a hydrophobic environment.

Example 3: Mutational Analysis of Hydrogen Bonds

The importance of hydrogen bonds for binding affinity was analysed by interaction analysis employing BMP-2 and BRIA_{EC} muteins with two kinds of amino acid substitutions. First, alanine substitutions were introduced to disrupt side chain bonds (see table 1 in example 2). Because the BMP-2 W28A mutein could not be isolated Trp 28 was substituted by phenylalanine whose side chain is also devoid of hydrogen bonding capacity. (One charged residue substitution was done in the BMP-2 S69R mutein.) Second, proline substitutions were introduced to abolish hydrogen bonding by the main chain amide group.

Interaction analysis of BMP-2 muteins with immobilised receptor BRIA_{EC} as represented in table 2 showed that substitutions W28F, D53A and Y103A had only small effects on binding affinity. A slightly higher reduction in binding affinity (17fold) was observed after charged residue insertion in the S69R mutein. Remarkably, the L51P and the D53P substitutions lead to dramatic lower affinities. The apparent dissociation constant K_D between BRIA_{EC} and L51P is more than 7100 times higher than that of BMP-2, and 53 times higher for D53P (Table 2 and Fig. 7). Both muteins have a similar drop in the affinity for the BRIIB receptor indicating that the same determinants of BMP-2 are used for binding of the two type I receptors. This suggests that the main chain hydrogen bonds disrupted in the two proline muteins are major binding determinants, whereas the side chain bonds of W28, D53, S69 and Y103 are of minor importance for binding to the BRIA receptor.

The affinity of the proline muteins for the type II receptor ARII is unaltered in comparison to the wild type (Table 2). The same has been observed for interaction with type II receptors Activin receptor IIB and BMP receptor II (data not shown). This confirms that the gross structure of the mutein is unchanged, since the local structural alteration due to the substituted proline is not propagated to the juxtaposed epitope for type II receptor binding (knuckle epitope).

Table 2: Binding affinities between BMP-2 muteins and the receptor ectodomains BRIA, BRIB and ARII, as well as between BMP-2 wild type and BRIA_{EC} muteins.

Ligand	Immobilised receptor EC		
	BRIA	BRIB	ARII
	$K_{D(\text{Mut})}/K_{D(\text{WT})}$		
BMP-2	1 (=0,91 nM)	1 (=3,6 nM)	1 (=14 nM)
BMP-2 W28F	3,5	1,7	1,2
BMP-2 L51P	>7100	>920	2,3
BMP-2 D53A	0,91	0,34	1,2
BMP-2 D53P	53	10	1,2
BMP-2 S69R	17	n.d.	0,9
BMP-2 Y103A	7,8	3,2	1,4
Ligand	Immobilised receptor EC		
	BRIA Q86A	BRIA S90A	BRIA Q94P
	$K_{D(\text{mut})}/K_{D(\text{WT})}$		
BMP-2	86	2,6	3,1

In order to identify binding determinants of the BRIA receptor three ectodomain muteins were prepared. In the receptor Q94P mutein the backbone nitrogen amide donating in the wild type a hydrogen bond to BMP-2 Ser 69 backbone carbonyl has been disrupted. The receptor Q86A mutein is devoid of the side chain carboxyamido group that in the wild type forms H-bond with BMP-2 L51 backbone amide and carbonyl (Fig. 2). The S90A substitution abolishes the serine hydroxyl group that donates a hydrogen bond to the BMP2 Val 26 backbone carbonyl. All receptor muteins could be highly purified after *E. coli* expression similar as the wild type ectodomain. When immobilised to the biosensor the receptor Q94P mutein bound BMP-2 with an only 3 times reduced affinity compared to the wild type ectodomain (Table 2) indicating that the main chain/main chain bond lost in the mutein contributes only marginally to binding. A similarly small change in affinity is observed in the S90A mutein.

The receptor Q86A substitution, however, resulted in an 86-fold increased K_D during BMP-2 interaction. Such a large decrease in affinity was however expected, since the BMP-2 L51P

mutein has already demonstrated that the affected H-bond represents a major binding determinant (see above). Most importantly, the hot spot identified by the mutational analysis coincides with the hot spot surmised by the analysis of the residual fractional accessibility of the engaged donor and acceptor groups (Fig. 1C). The loss in affinity in the receptor Q86A mutein is less severe than in the BMP-2 L51P mutein (Table 2). Neighbouring side chains in the receptor, e.g. Lys 79, or Glu 81, or Asp 89, might compensate partially for the carboxamide group of Gln 86 lost (Fig. 1b). On the other hand, the proline introduced in BMP-2 L51P causes steric hindrance or disturb nearby bonds by local structural rearrangements, e.g. between the BMP-2 Asp 53 backbone amide and the receptor Cys 77 carbonyl, and thereby would aggravate the effect of the substitution (Fig. 2).

Example 4: Structure of Proline BMP-2 Mutein L51P

Comparing the large decrease in binding affinity of BMP-2 L51P with that observed for BRIA_{EC} Q86A suggests that the loss in binding affinity might result not only from the loss of a single hydrogen bond. In addition larger structural changes in the binding epitope might disrupt the protein interaction. Therefore, BMP-2 L51P and also D53P muteins were submitted to crystallisation trials. BMP-2 L51P could be crystallised under the same conditions as wild type BMP-2, with identical crystal parameters, e.g. the same space group, R32, and almost identical cell constants as observed for crystals of wild type BMP-2. Apparently, the proline residue can be accommodated in place of Leu 51 whose hydrophobic side chain points into the protein core of BMP-2. The mutein D53P did not crystallise under those conditions, crystals of D53P growing from either ethanol or 2-methyl-2,4-pentandiol diffracted only to very low resolution and were not further analysed.

Diffraction data for BMP-2 L51P were obtained at room temperature to a resolution of 2.7 Å. Apart from the site of mutation the structures of BMP-2 wild type and L51P are basically identical within the error boundaries of the structure determination method depicted in table 3; the r.m.s. deviation for all C_α atom positions is 0.6 Å. Clear changes are observed in the pre-helix loop comprising residues Pro 48 to His 54 carrying also the site of mutation at position 51. For residues Pro 48, Phe 49, Pro 50, Leu/Pro51, Ala 52, and His 54 the distances between the C_α-position of the wild type and mutein exceeds 1 Å, with the largest difference of 1.8 Å for the

C_{α} of Ala 52 (table 3). Since the overall positional error for both structures, BMP-2 wild type and L51P, is about 0.4 as calculated from σ_A -plot, the change in conformation for the pre-helix loop might be considered small but significant.

Table 3: Processing and refinement statistics for BMP-2 – BRIA complex and BMP-2 L51P.

Crystals and data processing	BMP-2 – BRIA complex	BMP-2 L51P
Beamline	X06SA SLS	home source
Wave length (Å)	0.918	1.541
Space group	P65	R32
Unit cell	$a = b = 107.49 \text{ \AA}$, $c = 102.28 \text{ \AA}$, $\alpha = \beta = 90^\circ, \gamma = 120^\circ$	$a = b = 94.29 \text{ \AA}$, $c = 102.88 \text{ \AA}$, $\alpha = \beta = 90^\circ, \gamma = 120^\circ$
Resolution (Å)	20.0 – 1.86 (1.91 – 1.86)	20.0 – 2.65 (2.82 – 2.65)
Number of collected reflections	247856 (21558)	19336 (615)
Number of unique reflections	54306 (7758)	5624 (377)
Completeness	96.8 (94.9)	93.5 (72.3)
Multiplicity	4.6 (2.8)	3.4 (1.6)
Rsym (%) for all reflections	8.3 (55.6)	6.0 (29.3)
$\langle \text{Intensity}/\sigma \rangle$	4.4 (1.2)	8.8 (2.4)
Refinement statistics		
Rcryst (%)	20.7 (36.6)	21.5 (33.2)
Rfree (%) (test set 5%)	22.9 (39.6)	23.5 (33.6)
r.m.s. deviation		
Bonds (Å)	0.017	0.006
Angles (deg.)	1.537	1.226
Dihedrals (deg.)	27.334	24.733
Impropers (deg.)	1.388	0.910
Average B-Factor (Å ²)	36.1	64.6
Coordinate error (cross-validated sigma) (Å)	0.3 (0.3)	0.4 (0.4)
Procheck analysis		
Residues in most favored region (%)	87.2	85.2
Residues in additional allowed region (%)	11.3	13.6
Residues in generously allowed region (%)	1.5	1.1
Residues in disallowed region (%)	0.0	0.0

This change may be due to altered backbone geometry for the proline residue compared to that for wild type leucine. However, comparing the structure of BMP-2 in its unbound and bound conformation, the differences between the C_{α} positions are of the same magnitude as observed between BMP-2 wild type and mutein L51P, showing that the conformation of the pre-helix loop is neither preformed nor fixated, and adapts on the receptor surface upon complex formation.

The side chain of Pro 51 is oriented as Leu 51 in wild type BMP-2, indicating that the packing of the hydrophobic core is not affected by the mutation. Additionally, exchange of Leu 51 to proline does not alter the orientation of the backbone carbonyl of residue 51 therefore one hydrogen bond of residue 51 with BRIA Gln 86 might still be formed in the complex (Figs. 2,3A). Considering the rather small structural differences introduced in the unbound proteins by the mutation the large change in binding affinity seems puzzling.

A superposition of the structure of L51P and the complex of BMP-2 - BRIA_{EC} reveals that the ring of the proline residue occupies the volume formerly used for the hydrogen bond between the BMP-2 Leu 51 amide and the side chain carbonyl of BRIA_{EC} Gln 86 (Fig. 3A). The larger spatial requirement for the proline ring introduces a steric hindrance between the side chains of BMP-2 Pro 51 and BRIA_{EC} Gln 86 (Fig. 3A). To accommodate Pro 51 its side chain needs to be pushed further back. Due to the rigid ring structure which basically fixes the amide nitrogen with respect to the ring orientation this corresponds to a rotation around the ϕ backbone torsion angle moving the backbone carbonyl of Pro 50 towards the side chain of BRIA_{EC} Gln 86 if the ring structure is pushed back. Consequently, by removing one steric hindrance another one is introduced possibly explaining the large loss in binding energy by the mutation L51P.

Example 5: Proline Muteins and biological activity

The biological activity of the BMP-2 proline muteins was determined by dose-dependent induction of alkaline phosphatase (ALP) activity using C2C12 cells as well as the slightly more sensitive ATDC5 cell line. The dose of wild type BMP-2 resulting in 50% of the maximal response (ED₅₀) was 25nM in C2C12 cells. Using ATDC5 cells the ED₅₀ was 8nM showing the increased responsiveness of these cells. In contrast, BMP-2 L51P has no measurable biological activity in C2C12 (> 1600nM) or in the ATDC5 cells (> 1500nM). The BMP-2 D53P mutein shows ED₅₀ values of 330nM and 140nM for C2C12 and ATDC5 cells, respectively. These values are about 13 to 17 times higher compared to the ED₅₀ value of wild type BMP-2. Thus, the different reduction of BRIA receptor affinity in the two BMP-2 proline muteins parallels the loss in biological activity (Fig. 4).

Since the proline substitution of BMP-2 Leu 51 is affecting only the interaction with the type I receptor while the affinity for the type II receptor is unaltered, the L51P mutein might principally function as a dominant-negative inhibitor. However, this was not observed when concentrations up to 250 nM of the mutein were applied to cells stimulated with 20 nM BMP-2 (data not shown). In comparison, the basal ALP activity in ATDC5 cells is inhibited by the antagonistic BMP-2 A34D mutein (Kirsch et al., 2000a), but not by L51P (Fig. 4A).

Example 6: Proline Muteins as antagonists of binding proteins

The lack of BMP-2 L51P to bind to BRIA_{EC} raised the question, whether the interaction with other binding proteins might be also affected by the proline substitution. Of particular interest is Noggin that binds BMPs with high affinity and whose structure in complex with BMP-7 has been recently determined (Groppe et al., 2002). Another important binding protein is Chordin, that interacts with BMP-2 by means of two cysteine-rich domains. Still another type of binding protein represents Gremlin that interacts with BMP-2 via a so-called DAN domain. Each of the three proteins was immobilised at the surface of a biosensor and analysed for BMP-2 mutein interaction as depicted in table 4.

Table 4: Binding affinities between BMP-2 proline muteins and Noggin, Chordin, and Gremlin (Biacore interaction analysis).

Ligand analyte	Immobilised binding protein			
	Noggin	Chordin CR1	Chordin CR3	Gremlin
		$K_D(\text{mut})/K_D(\text{WT})$		
BMP-2	1 (=1,9 nM)	1 (=61 nM)	1 (=67 nM)	1 (=22 nM)
BMP-2 D53P	1,3	0,7	1,1	0,7
BMP-2 L51P	0,27	0,85	0,9	0,55

The dissociation constants K_D of the two BMP-2 proline muteins for the complex with Noggin were similar to those of BMP-2 wild type and ranged from 0.5 to 2.5 nM. Thus, BMP-2 Leu 51 and Asp 53 are not the main binding determinants for Noggin, despite the fact that the N-

terminal amino acids of Noggin have been shown to be in contact with the BMP-7 region corresponding to BMP-2 Leu 51 (BMP-7 Leu 75) and Asp 53 (BMP-7 Ser 77, pre-helix loop) and that Noggin prevents BRIA binding to BMP-2.

The affinity of BMP-2 for Chordin domain CR1 and CR3 as well as for Gremlin is 10 to 30 times lower than that for Noggin (table 4). Again a similar dissociation constant K_D is found for BMP-2 wild type or the two proline mutoins, indicating that the binding of these proteins is not affected by the proline substitutions. Thus the BMP-2 epitopes for both binding proteins differ from that for the BRIA receptor.

Example 7: Proline mutoin L51P releases Noggin inhibition in C2C12 cells

BMP-2 induces in starved promyeloblastic cell line C2C12 the expression of alkaline phosphatase. This BMP response is inhibited to background levels by equimolar concentration of Noggin-Fc fusion protein (Fig. 5). The inhibition of BMP-2 activity by Noggin is released in a dose dependent manner by the L51P mutoin. At roughly equimolar concentrations of the mutoin L51P and Noggin the inhibitory effect of Noggin is neutralized to more than 80%. This indicates that the receptor-dead L51P mutoin represents an efficient inhibitor of the Noggin protein. This result is in accordance with the physical binding studies showing disruption of type I receptor binding and conservation of Noggin binding after the BMP-2 L51P substitution.

References

In order to avoid unnecessary reference a comprehensive list of the references mentioned herein is presented in the following. It is to be understood that the disclosure of any of these references is incorporated herein by reference.

Balemans, W. and Van Hul, W. (2002) Extracellular regulation of BMP signaling in vertebrates: a cocktail of modulators. *Dev Biol*, **250**, 231-250.

Bogan, A.A. and Thorn, K.S. (1998) Anatomy of hot spots in protein interfaces. *J Mol Biol*, **280**, 1-9.

Boulanger, M.J., Chow, D.C., Brevnova, E.E. and Garcia, K.C. (2003) Hexameric structure and assembly of the interleukin-6/IL-6 alpha-receptor/gp130 complex. *Science*, **300**, 2101-2104.

Brunger, A.T. (1992) Free R value: a novel statistical quantity for assessing the accuracy of crystal structures. *Nature*, **355**, 472-475.

Brunger, A.T., Adams, P.D., Clore, G.M., DeLano, W.L., Gros, P., Grosse-Kunstleve, R.W., Jiang, J.S., Kuszewski, J., Nilges, M., Pannu, N.S., Read, R.J., Rice, L.M., Simonson, T. and Warren, G.L. (1998) Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr D Biol Crystallogr*, **54** (Pt 5), 905-921.

Chakrabarti, P. and Janin, J. (2002) Dissecting protein-protein recognition sites. *Proteins*, **47**, 334-343.

Collaborative Computational Project, N. (1994) The CCP4 Suite: Programs for Protein Crystallography. *Acta Crystallogr sect D*, **50**.

de Vos, A.M., Ultsch, M. and Kossiakoff, A.A. (1992) Human growth hormone and extracellular domain of its receptor: crystal structure of the complex. *Science*, **255**, 306-312.

Evans, P.R. (1993) Data reduction. *Proceedings of CCP4 Study Weekend*, pp. 114-122.

Greenwald, J., Groppe, J., Gray, P., Wiater, E., Kwiatkowski, W., Vale, W. and Choe, S. (2003) The BMP7/ActRII extracellular domain complex provides new insights into the cooperative nature of receptor assembly. *Mol Cell*, **11**, 605-617.

Groppe, J., Greenwald, J., Wiater, E., Rodriguez-Leon, J., Economides, A.N., Kwiatkowski, W., Affolter, M., Vale, W.W., Belmonte, J.C. and Choe, S. (2002) Structural basis of BMP signalling inhibition by the cystine knot protein Noggin. *Nature*, **420**, 636-642.

Hage, T., Sebald, W. and Reinemer, P. (1999) Crystal structure of the interleukin-4/receptor alpha chain complex reveals a mosaic binding interface. *Cell*, **97**, 271-281.

Hart, P.J., Deep, S., Taylor, A.B., Shu, Z., Hinck, C.S. and Hinck, A.P. (2002) Crystal structure of the human TbetaR2 ectodomain--TGF-beta3 complex. *Nat Struct Biol*, **9**, 203-208.

Kirsch, T., Nickel, J. and Sebald, W. (2000a) BMP-2 antagonists emerge from alterations in the low-affinity binding epitope for receptor BMPR-II. *Embo J*, **19**, 3314-3324.

Kirsch, T., Nickel, J. and Sebald, W. (2000b) Isolation of recombinant BMP receptor IA ectodomain and its 2:1 complex with BMP-2. *FEBS Lett*, **468**, 215-219.

Leslie, A.G.W. (1992) Recent changes to the MOSFLM package for processing film and image plate data. *Joint CCP4 + ESF-EAMCB Newsletter on Protein Crystallography*, **26**.

McDonald, I.K. and Thornton, J.M. (1994) Satisfying hydrogen bonding potential in proteins. *J Mol Biol*, **238**, 777-793.

Murshudov, G.N., Vagin, A.A. and Dodson, E.J. (1997) Refinement of Macromolecular Structures by the Maximum-Likelihood Method. *Acta Crystallogr sect D*, **D53**, 240-255.

Ruppert, R., Hoffmann, E. and Sebald, W. (1996) Human bone morphogenetic protein 2 contains a heparin-binding site which modifies its biological activity. *Eur J Biochem*, **237**, 295-302.

Syed, R.S., Reid, S.W., Li, C., Cheetham, J.C., Aoki, K.H., Liu, B., Zhan, H., Osslund, T.D., Chirino, A.J., Zhang, J., Finer-Moore, J., Elliott, S., Sitney, K., Katz, B.A., Matthews, D.J., Thompson, T.B., Woodruff, T.K. and Jardetzky, T.S. (2003) Structures of an ActRIIB:activin A complex reveal a novel binding mode for TGF-beta ligand:receptor interactions. *Embo J*, **22**, 1555-1566.

Winn, M.D., Isupov, M.N. and Murshudov, G.N. (2001) Use of TLS parameters to model anisotropic displacements in macromolecular refinement. *Acta Crystallogr D Biol Crystallogr*, **57**, 122-133.

Xu, D., Tsai, C.J. and Nussinov, R. (1997) Hydrogen bonds and salt bridges across protein-protein interfaces. *Protein Eng*, **10**, 999-1012.

The features of the present invention disclosed in the specification, the claims and/or the drawings may both separately and in any combination thereof be material for realizing the invention in various forms thereof.